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Transgenic Pigs Expressing Human Decay-Accelerating Factor Regulated by Porcine MCP Gene Promoter

HIROSHI MURAKAMI,[1]* HIROSHI NAGASHIMA,[2] YOICHI TAKAHAGI,[1] SHUJI MIYAGAWA,[3] TATSUYA FUJIMURA,[1] KOJI TOYOMURA,[4] RIE NAKAI,[3] MAKO YAMADA,[1] TAKASHI KURIHARA,[3] TAMOTSU SHIGEHISA,[1] MASARU OKABE,[5] TSUKASA SEYA,[6] RYOTA SHIRAKURA,[2] AND TAROH KINOSHITA[7]

[1]*The Animal Engineering Research Institute, Tsukuba, Ibaraki*
[2]*Laboratory of Reproduction Engineering, Meiji University, Kawasaki, Kanagawa*
[3]*Division of Transplantation, Department of Regenerative Medicine, Osaka University Graduate School of Medicine, Suita, Osaka*
[4]*R & D Center, Nippon Meat Packers Inc., Tsukuba, Ibaraki*
[5]*Genome Information Research Center, Osaka University, Suita, Osaka*
[6]*Department of Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Higashinari, Osaka*
[7]*Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan*

ABSTRACT Porcine membrane cofactor protein (pMCP) is abundantly expressed throughout the body with particularly strong expression on the vascular endothelia. Previous studies demonstrated that the promoter of the pMCP gene induced efficient expression of a human complement regulatory protein, decay-accelerating factor (DAF; CD55), in transgenic mice. In the present study, we tried to produce transgenic pigs with two hybrid genes, 0.9/hDAF and 5.4/hDAF, which were composed of human DAF (hDAF) gene regulated under pMCP promoters of different lengths (0.9 and 5.4 kb). Five live founder transgenic pigs were obtained only with the 0.9/hDAF construct. Although, four founder pigs transmitted the transgene to the second generation, the transmission rates varied among founders. We examined the expression of hDAF in tissues of descendants of two lines (Dm1 and Dm4). Human DAF specific RNAs were confirmed by an RT-PCR analysis in all organs examined. Levels of hDAF protein in the organs from the descendants of Dm1 line were higher than those in the corresponding human organs as determined by enzyme-linked immunosorbent assay. Immunohistochemical studies showed that the tissue distribution of hDAF in the descendants of both lines was similar to that of endogenous pMCP. The expression level of hDAF on the vascular endothelial cells in Dm1 line was twice that on the corresponding human cells. We tested whether proinflammatory cytokines upregulate an efficiency of pMCP promoter on hDAF expression in transgenic pigs. Although the expression of hDAF on the human endothelial cells increased with a combination of cytokines, tumor necrosis factor α and interferon-γ, no cytokine-induced upregulation was seen in the cells of transgenic pigs. The endothelial cells from transgenic pigs exhibited high resistance to the human serum-mediated cytolysis. *Mol. Reprod. Dev. 61: 302–311, 2002.* © 2002 Wiley-Liss, Inc.

Key Words: porcine MCP; human DAF (CD55); transgenic pig; xenotransplantation

INTRODUCTION

The major obstacles to xenotransplantation of vascularized organs in discordant species combinations, such as pig to human, are hyperacute rejection (HAR) and acute vascular rejection (AVR) (Lawson and Platt, 1996). HAR leads to severe graft destruction, such as edema, hemorrhage, thrombosis, and necrosis within minutes to hours (Platt et al., 1991). In the pathogenesis of HAR, the binding of xenoreactive antibodies to donor vascular endothelial cells and following activation of recipient's complement are critical factors (Dalmasso et al., 1992). To prevent recipient's complement activation, donor pigs that are transgenic for human complement regulatory proteins (CRPs) such as decay accelerating factor (DAF, CD55) (Cozzi et al., 1994), CD59 (Fodor et al., 1994), and membrane cofactor protein (MCP, CD46) (Adams et al., 2001), have been produced. Pig-to-baboon transplantation experiments using those transgenic pigs showed that HAR was prevented (Schmoeckel et al., 1998). In AVR that is characterized by hemorrhage, thrombosis, and a mononuclear cell infiltration into donor organs,

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*Correspondence to: Hiroshi Murakami, Animal Engineering Research Institute, 3-3 Midorigahara, Tsukuba, Ibaraki, 300-2646. Japan. E-mail: murakami@rdc.nipponham.co.jp

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activated T lymphocytes, macrophages, and NK cells play roles (Platt et al., 1998). It was shown that AVR could occur independently of complement activation following binding of xenoreactive antibodies to vascular endothelia of the grafts. To prevent AVR, donor pigs should be manipulated to reduce the major xenoepitope Gal α (1,3)Gal (Galili et al., 1987) and to regulate activation of inflammatory cells like NK cells. Disruption of α 1,3galactosyltransferase gene by homologous recombination would be effective; however, the method is yet to be developed in pig. Alternatively, an overexpression of other glycosyltransferase genes, such as α 1,2-fucosyltransferase-that competes with α 1,3galactosyltransferase for its substrate-would reduce Gal α (1,3)Gal epitope (Sandrin et al., 1995). It was also reported that expression of HLA molecules on pig cells effectively inhibited human NK cell-mediated cytotoxicity in vitro (Sasaki et al., 1999). The potential benefit of combined expressions of the genes like CRPs and glycosyltransferases has been demonstrated in mice (Cowan et al., 1998a).

Several gene promoters have been tested in producing transgenic pigs for xenotransplantation. Murine H-2 gene and human intercellular adhesion molecule 2 promoters have been used to express human CRPs in mice and pigs (Fodor et al., 1994; McCurry et al., 1995; Cowan et al., 1998b). However, the expression levels were insufficient particularly in pigs (Byrne et al., 1997; Nottle et al., 1999). These results suggest that the regulation mechanisms for transgene expression are not the same in mice and pigs. Minigene and genomic DNA exhibited sufficient expression levels of human CRPs in pigs (Cozzi et al., 1997; Chen et al., 1999; Adams et al., 2001). However, the minigene and genomic DNAs having homologous promoter of the coding genes express the genes in the same way that the genes are originally expressed in vivo. Therefore, promoters that express heterologous genes on vascular endothelia or other clinically important tissues, such as pancreatic islets in pigs, should be developed.

We and others identified pig homologue of human MCP and studied its activity and tissue distribution (Toyomura et al., 1997; van den Berg et al., 1997). Immunohistochemical analysis with anti-pMCP revealed that pig MCP (pMCP) is widely and abundantly expressed in all tissues examined with particularly strong staining on the vascular endothelia (Perez de la Lastra et al., 1999). We identified a promoter region of the pMCP gene and found that it supports high level expressions of foreign proteins in porcine aorta endothelial (PAE) cells. We also produced transgenic mice with pMCP promoters of different lengths and demonstrated that both long and short forms expressed human DAF at high levels in various mouse tissues (Murakami et al., 2000).

In the current experiment, we conducted further studies to confirm effectiveness of the pMCP promoter in pigs. We produced lines of transgenic pigs expressing hDAF at higher levels than human cells in a tissue distribution similar to endogenous pMCP.

MATERIALS AND METHODS

Gene Constructs

We used two hybrid genes in which 0.9- and 5.4-kb upstream sequences from the pMCP gene were each ligated with hDAF minigene (referred to as 0.9/hDAF and 5.4/hDAF) as described previously (Murakami et al., 2000). Briefly, the hDAF cDNA including first intron was ligated at 59 bp downstream of the transcriptional start site within the first exon of the pMCP gene − 5400 to + 59 or − 841 to + 59; The DDBJ/EMBL/GenBank accession number of nucleotide sequence of the 5′-flanking region of the pMCP gene is AB025019. We digested the plasmids with *Not*I and *Eco*47III to remove the vector sequences, and purified the DNA fragments for microinjection with spin columns (ULTRAFREE®-MC, Millipore Co. Bedford, MA).

Generation and Breeding of Transgenic Pigs

We used prepubertal Landrace, Large White, and crossbred gilts (Landrace/Large White × Duroc) as embryo donors and recipients. Methods of superovulation for gilts were described previously (Takahagi et al., 1999). Embryo donors were artificially inseminated, and embryos were collected 50–54 hr after the hCG injection. Embryos were centrifuged at 12000*g* for 8 min to visualize the pronuclei and microinjected with about several thousands copies of each hybrid gene. Microinjected embryos were then transferred to unmated synchronized recipients or the embryo donors (donor-recipients) (Pursel and Wall, 1996). Transgenic pigs were identified by PCR and/or Southern blot analysis with genomic DNA extracted from the tail tips of the newborn pigs. Founder transgenic pigs and their transgenic descendants were bred with non-transgenic boars or gilts to obtain the second and third generations. Copy numbers of the transgenes in pigs of the third generation were estimated by a DNA dot blot analysis as described previously (Murakami et al., 2000).

RT-PCR

One microgram of total RNA prepared with Isogen (Nippon Gene Co., Japan) was reverse-transcribed using a first-strand cDNA synthesis kit (GIBCO-BRL, Rockville, MD). We used primers 5′-gtgcctgccggccg-tgtggggt and 5′-tccataatggtcacgttcccctg to amplify 652 bp of hDAF sequence. One hundred nanograms of the first-stranded cDNAs were used for PCR with an amplification condition; 30 cycles of denaturation at 94°C for 30 sec, and annealing and extension at 68°C for 3 min.

Immunohistochemical Staining

Tissues were embedded in OCT compound (Miles Inc., Elkhart, IN) and frozen in dry ice-ethanol. Tissue sections (8 μm) were mounted on poly-L-lysin-coated slides, air dried, and fixed in acetone. Endogenous peroxidase was removed by incubation with 0.02% H_2O_2 in

PBS. Endogenous tissue biotin was blocked using Blocking kit (Vector Laboratories, Inc., Burlingame, CA). Tissues were stained with a biotinylated mAb to hDAF (10 μg/ml of IA10), followed by development with an avidin–biotin complex horseradish peroxidase method (VECTASTEIN ABC kit, Vector Laboratories). Peroxidase staining was carried out using diaminobenzidine reagent set (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Tissues were counterstained with hematoxylin.

Cells and Culture

Pig endothelial cells from several lines of transgenic and nontransgenic pigs were isolated by scraping the aorta with a spatula and culturing in DMEM containing 10% FBS with L-glutamine (GIBCO-BRL) and penicillin/streptomycin (Meiji, Tokyo, Japan). Human umbilical arterial endothelial cells (HAECs) purchased from Cell Systems Corporation (Kirkland, WA) were cultured with CSC-Complete Medium Kit according to a manufacturer's recommended protocol. Human umbilical vein endothelial cells (HUVECs, a line ID; IF0500271) were provided from Institute for Fermentation Osaka. HUVECs were cultured in Ham F12 medium (Dainippon Pharm. Co, Osaka, Japan) supplemented with 10% FBS, 50 μg/ml of endothelial cell growth supplement (Becton Dickinson Labware, Bedford, MA) and 100 μg/ml of heparin sodium (Wako Pure Chemical Industries, Ltd. Osaka, Japan). In each experiment, pig endothelial cells at passage 2–5 were used. Splenocytes from transgenic and nontransgenic pigs were prepared by sieving and incubated in distilled water for 30 sec to lyse RBCs followed by an addition of the same volume of 2 × PBS and centrifugation. This operation was repeated until RBCs were deleted from the cell pellet. The cells were washed twice with PBS and suspended in PBS.

Flow Cytometric Analysis

Cells were suspended in PBS containing 1% bovine serum albumin and 0.1% sodium azide (FACS buffer) and incubated with a biotinylated anti-hDAF mAb (10 μg/ml of IA10) in 100 μl on ice for 30 min. They were then washed twice with FACS buffer and incubated with 10 μg/ml of streptavidin-conjugated phycoerythrin (Biomeda Co., Foster, CA) in 100 μl on ice for 30 min. They were washed again twice, resuspended in FACS buffer containing 10% formaldehyde, and analyzed by a FACScan (Becton Dickinson).

Immunoprecipitation and Western Blotting Analysis

One hundred micrograms of liver from transgenic and nontransgenic pigs, and 5×10^8 of human peripheral red blood cells (PRBCs) were homogenized in 1 ml of a lysis solution (20 mM Tris-HCl (pH 7.5), 1% NP-40, 1 mM PMSF, 5 mM EDTA, 10 mM iodoacetoamide, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin), followed by centrifugation to remove insoluble materials. The supernatants were used in the following procedures. Protein G-Sepharose (100 μl beads) was incubated with anti-hDAF monoclonal antibody (IIH6: 50 μg) at room temperature for 1 hr, and washed with PBS–BSA (1 mg/ml: proteinase-free) three times. The lysates were incubated with the IIH6-Protein G Sepharose (20 μl beads/1ml lysate) at 4°C for 1 hr and again washed twice with PBS–BSA. The bound hDAF molecules were eluted from the beads by incubation for 5 min at 80°C with 100 μl of sample buffer consisting of 5% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol and 0.01% bromphenol blue. After SDS–PAGE under non-reducing conditions on a 5–20% gel, proteins were blotted onto polyvinylidene difluoride membrane using a wet Western transfer apparatus (Bio-Rad). The membrane was blocked in 20 mM Tris-HCl (pH 7.5), 0.1% Tween 20 with 5% blocking reagent (RPN2108 ECL Western blotting analysis system: Amersham Pharmacia Biotech) for 1 hr at room temperature and reacted with either anti-hDAF mAb (VIIIA7: 4 μg/ml) or irrelevant IgG mAb (4 μg/ml) for 1 hr at room temperature. Western blot was developed by a chemiluminescent detection system (RPN2108 ECL Western blotting analysis system).

Enzyme-Linked Immunosorbent Assay (ELISA)

Human organ specimens were obtained from a cadaver 6 hr post mortem with the informed consent for this study of the patient involved. Tissue blocks were excised, snap frozen in liquid nitrogen, and stored at −95°C until use. Tissues were homogenized in the NP-40 lysis buffer referred in the former heading and incubated at 4°C for 1 hr. The tissue lysates were centrifuged, and the supernatants were deep frozen and kept at −95°C until use. To the microtiter wells coated with IIH6 mAb at 5 μg/ml, triplicates of 100 μl tissue lysates prepared to achieve protein concentration at 1 mg/ml were added and incubated for 4 hr at 4°C. After washing with PBS containing 0.05% Tween 20 (PBS-T), incubation with 100 μl of biotinylated IA10 mAb at 1 μg/ml for 2 hr at 4°C, washing with PBS-T again, and following incubation with avidin conjugated alkaline phosphatase (ZYMED) for 30 min were carried out. The reaction was developed with Lumi-Phos® 530 (Wako Pure Chemical Industries, Ltd.) and enzyme activities were estimated with a microplate fluorometer (Fluoroskan Ascent FL®, Thermo Labsystems, Helsinki, Finland).

Cytokine Induced hDAF Expression

Cells (2×10^5) were seeded in 60-mm petri dishes. Eight hours later, recombinant human IL-1β, or tumor necrosis factor α (TNF-α) and interferon-γ (INF-γ) were added in cultures at 10 ng/ml, 10 ng/ml, and 500 U/ml respectively. Forty-eight hours later, we harvested the cells using trypin/EDTA, and measured the hDAF expressions by flow cytometric analysis. All the cytokines were purchased from PEPRO TECH EC Ltd. (London, UK).

Complement-Mediated Cell Lysis Assay

This assay was performed using an MTX "LDH" kit (Kyokuto, Tokyo, Japan). The endothelial cells from transgenic pigs were plated at 2×10^4 cells per well in flat-bottomed gelatin-coated 96-well trays, 1 day prior to assay. Fifteen hours after plating the cells, the wells were washed twice with serum-free DMEM to remove the lactate dehydrogenase (LDH), which is present in fetal calf serum, and incubated with several concentrations of normal human serum (NHS), which had been diluted with DMEM. The plates were incubated for 2 hr at 37°C and the released LDH was then measured. The percent cytotoxicity was calculated using the formula;

$$\text{Cytotoxicity} = \{(E - N - S)/(M - N - S)\} \times 100$$

where E is the experimentally observed release of LDH activity from the target cells, N; the LDH activity in each concentration of NHS, S; the spontaneous release of LDH activity from target cells incubated in the absence of NHS, and M; the maximal release of LDH activity, as determined by sonication. The spontaneous release of LDH activity from cells was less than 5%, compared to the maximal release obtained by sonication.

Statistics

The Student *t*-test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Generation of Transgenic Pigs and Transmission of the Transgenes

We microinjected 0.9/hDAF DNA into 362 pig ova and obtained five transgenics in 43 newborns. There was no transgenic pig in 76 newborns obtained from 1122 ova microinjected with 5.4/hDAF DNA. The four live founder transgenic pigs were bred and mated with non-transgenic boars or gilts. Table 1 shows the numbers of descendants from each founder pig. All founders transmitted the transgene to the second generation; however, the transmission rates varied among lines. Founders of Dm2 and Dm5 transmitted the transgene to only one descendant in 31 and 23 offspring, respectively, whereas Dm4 founder transmitted the transgene to all 15 offspring. The transgenic rate in the third generation of Dm4, 84%, was much higher than the expected rate of 50%. The transgenic rate of Dm1 was lower than 50% in the second generation but was as expected in the third generation. Dm1 descendants had about 20 copies of the transgene in the third generation. We obtained two different copy numbers, about 10 and 50, in Dm4 descendants (data not shown), suggesting more than one transgene integration site. We used Dm1 and Dm4 descendants of the third generation in subsequent studies.

Expression of hDAF in Various Tissues of Transgenic Pigs

mRNA expression in the organs from transgenic pigs. We determined hDAF mRNA expression in various tissues from transgenic and non-transgenic littermates with RT-PCR. All transgenic organs analyzed, namely, heart, lung, kidney, liver, skin, spleen, and cerebrum, expressed hDAF mRNA as shown by generation of the specific 652 bp DNA fragment (Fig. 1, lanes 2, 4, 6, 8, 10, 12, and 14). Samples of a non-transgenic littermate did not generate specific PCR products (lanes 3, 5, 7, 9, 11, 13, and 15).

Tissue distribution of hDAF in transgenic pigs determined with immunohistochemistry. To determine hDAF expression profiles in various tissues from hDAF transgenic pigs, we made sections of heart, lung, kidney, liver, skin, spleen, pancreas, and cerebrum from transgenic and non-transgenic littermates and stained them with hDAF specific mAb. All sections of transgenic tissues were specifically stained, whereas the section of non-transgenic pigs gave only background staining. Vascular endothelia of capillaries, venules and arterioles, and nerves in all transgenic tissues examined were intensely stained (Fig. 2). Other parts of tissues were also specifically stained (Table 2). In heart, capillaries in myocardium were intensely stained (Fig. 2A). Myocytes were weakly stained, whereas atrial myocardium was stained stronger than ventricular myocardium. In aorta, endothelial cells and smooth muscle cells in tunica media were intensely stained (Fig. 2B). In kidney, glomeruli and interlobular vessels were intensely stained (Fig. 2C). Proximal renal tubules were faintly stained, whereas the distal tubules were less stained. In liver, interlobular arteries and veins, and its surrounding connective tissues were intensely stained, whereas the epithelial cells of bile ducts seemed not to be stained (Fig. 2D). Hepatocytes were faintly stained. In skin, keratinocytes in epidermis and vessels in dermis were intensely stained (Fig. 2E). In lung, the whole tissues

TABLE 1. Transmission Rates of the Transgenes in the Four Transgenic Pig Lines

		2nd generation		3rd generation	
Founders	Sex	Total	Transgenics	Total	Transgenics
Dm1	Female	47	13	47	25
Dm2	Female	31	1		
Dm4	Female	15	15	25	21
Dm5	Male	23	1		

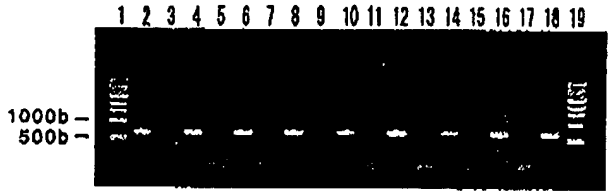

Fig. 1. RT-PCR analysis of hDAF mRNA expression in various tissues from transgenic pigs. Lane 2, heart; Lane 4, lung; Lane 6, kidney; Lane 8, liver; Lane 10, skin; Lane 12, spleen; Lane 14, cerebrum from a transgenic Dm1 pig; Lane 3, heart; Lane 5, lung; Lane 7, kidney; Lane 9, liver; Lane 11, skin; Lane 13, spleen; Lane 15, cerebrum from a non-transgenic littermate; Lane 16, endothelial cells from a transgenic littermate; Lane 17, endothelial cells from a non-transgenic littermate; Lane 18, HAECs. Lanes 1 and 19; 1 kb ladder markers. The hDAF specific 652 bp DNA fragment was detected in all transgenic and human samples.

including alveoli, bronchioles, and vessels were strongly stained. In pancreas, excretory ducts and vessels were intensely stained, but weak staining was observed in the exocrine acinar cells and islets.

Quantitative analysis of hDAF expression in the organs of transgenic pigs. For quantitative measurement of the hDAF expressions in different organs of transgenic pigs and nontransgenic littermates, we used ELISA and compared them with the endogenous hDAF expressions in equivalent human tissues (Fig. 3). The organs analyzed were from Dm1 and Dm4 descendants. A relatively constant expression was obtained in the organs from Dm1 descendants. Almost all the organs from Dm1 descendants analyzed were found to express amounts of hDAF comparable to or greater than those found in the equivalent human tissues. In contrast, the levels of hDAF expression varied considerably among individuals of Dm4 descendants except for the expression in liver.

We then analyzed the hDAF expression levels on the aorta endothelial cells and splenocytes by flow cytometry. The endothelial cells from Dm1 descendants expressed hDAF 1.5–2 times higher than human aorta ndothelial cells (Fig. 4). The splenocytes from Dm1 descendants also expressed hDAF as much as human peripheral lymphocytes. The similar expression intensity of hDAF was observed on the cells from Dm4 descendants (Fig. 6). We also analyzed hDAF expression levels on the PRBCs from descendants of both lines by flow cytometry. The PRBCs from 1-week-old descendants expressed hDAF higher than human PRBCs. However, the level was decreased to nearly 0 when the descendants reached 10 months old (data not shown). This downregulation of the hDAF expression was seen only with PRBCs.

Characterization of hDAF Protein Expressed in Transgenic Pigs

We analyzed the hDAF protein expressed in the transgenic pigs by Western blotting (Fig. 5). A 75-kD band corresponding to the molecular weight of endogenous hDAF was identified in both the transgenic liver extracts (lane 2) and human peripheral red blood cell extracts (lane 4). No equivalent band was detected in the liver extracts from non-transgenic littermates (lane 3).

Effects of Cytokines on hDAF Expression in the Transgenic Pigs

It was reported that hDAF expression is upregulated by proinflammatory cytokines, such as IFN-γ. Upregulation of CRPs on cells under inflammatory conditions would be beneficial. It is not known whether the promoter of pMCP is regulated by cytokines. We tested whether hDAF expression on the endothelial cells of transgenic pigs is regulated by cytokines. The cells from three transgenic and one nontransgenic descendants, and two human endothelial cell lines were stimulated with IL-1β or a combination of TNF-α and IFN-γ and subsequently analyzed by flow cytometry (Fig. 6). The hDAF was constitutively expressed on the resting endothelial cells of both human and the transgenic pigs. The levels of hDAF expression on the resting cells from transgenic pigs were approximately two-fold higher than human cells. The expressions on HAECs and HUVECs were increased up to two-fold with the combination of TNF-α and IFN-γ. No significant increase was observed on the cells of transgenic pigs with the cytokine combination. No significant change of the levels of hDAF expression was observed with IL-1β.

Functional Analysis of hDAF Expressed in the Transgenic Pigs

We demonstrated the protective effect of the hDAF protein synthesized in the transgenic pigs in human complement-mediated cell lysis assay. The endothelial cells from three transgenic descendants and a non-transgenic pig were subjected to the cell lysis assay. A non-transgenic pig endothelial cell line (WDB2), transfected with hDAF gene (Miyagawa et al., 1994), were used as a positive control cells expressing hDAF at the similar levels of the transgenic pig cells. The cells were cultured in the medium containing 20% or 40% of NHS followed by an LDH release assay. High death rates of the cells were observed in the non-treated, non-transgenic pig cells by adding NHS in the medium (Fig. 7). A significant decrease in the rates was observed in the cells from transgenic pigs and the hDAF expressing non-transgenic pig cells with any concentration of NHS.

DISCUSSION

To overcome early major rejections such as HAR and AVR as well as cell-mediated rejections in pig-to-human xenotransplantation, it would be necessary to modify pigs with genes effective for complement regulation, reduction of xenoantigens, and NK cell regulation. Such gene modifications require transgenic technology in pigs including cloning and homologous recombination techniques as well as accumulation of information about regulatory elements f genes useful to express heterologous genes in targeted tissues in

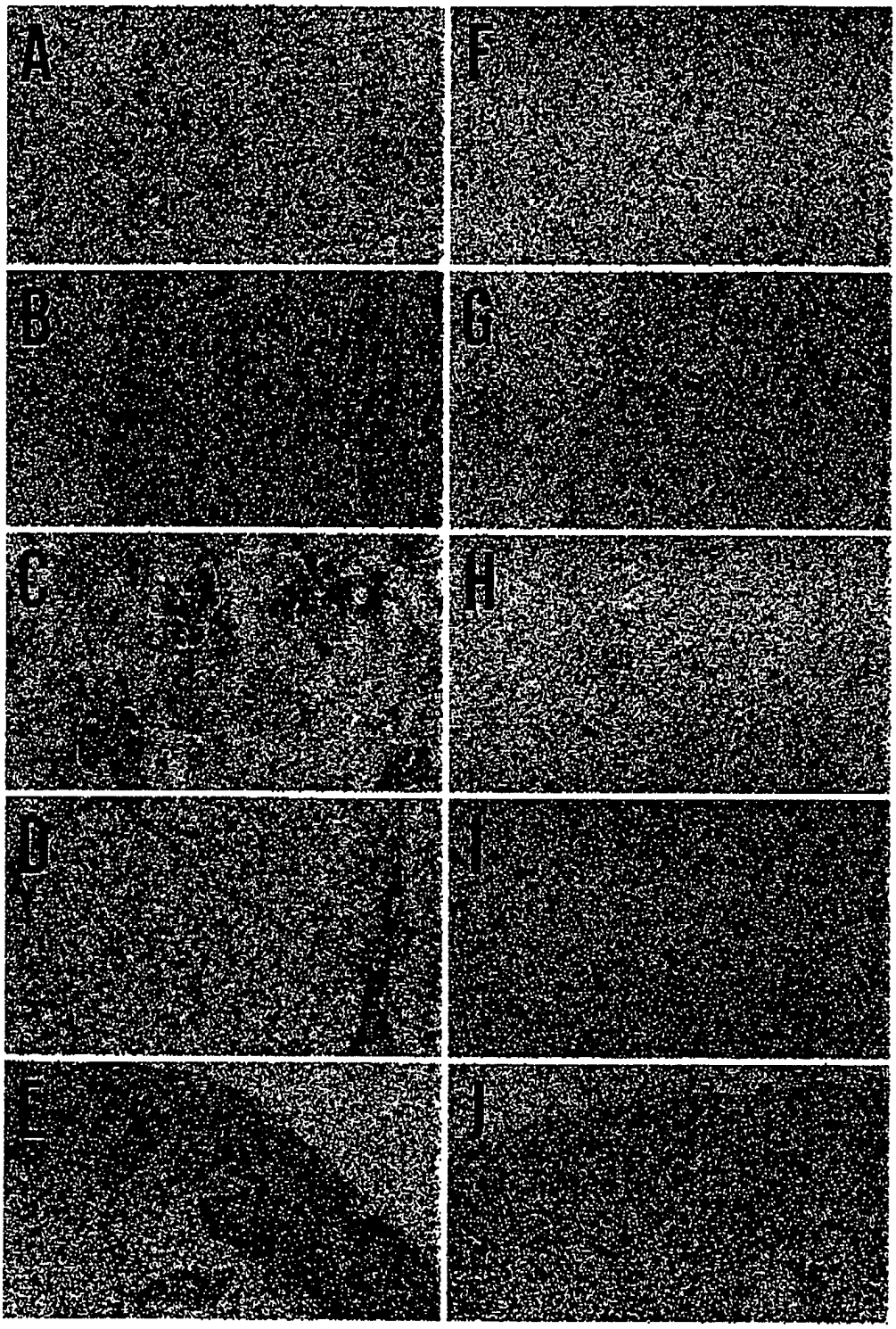

Fig. 2. Immunohistochemical staining for hDAF in various tissues from transgenic pigs. A peroxidase staining was performed as described in Materials and Methods. A to E, tissues from Dm1 transgenic descendants; F to J, tissues from the non-transgenic littermates. A and F, heart (Magnification 200 ×); B and G, aorta (200 ×); C and H, kidney (200 ×); D and I, liver (100 ×); E and J, skin (100 ×).

TABLE 2. Immunostaining Profile of Tissues in the Transgenic Pigs

Organs	Tissues	Intensity
Heart	Endomyocardium	++
	Atrial myocardium	++
	Ventricular myocardium	+
Kidney	Glomeruli	+++
	Proximal tubules	++
	Distal tubules	+/−
Liver	Hepatocytes	+
	Sinusoidal capillaries	++
	Interlobular arteries	+++
	Interlobular veins	+++
	Bile ducts	+/−
Pancreas	Exocrine gland cells	+/−
	Islets	+
Lung	Alveolar epithelia	++
	Bronchiolar epithelia	++
Cerebrum	Cerebral cortex	++
	Cerebral medulla	++
Skin	Epidermis	++
	Dermis	+
Spleen	Pulps	++
	Trabeculae	+
Aorta	Vascular endothelia	+++
	Smooth muscle	++
*	Capillary endothelia	+++
	Small arterie walls	+++
	Small vessel walls	+++
	Nerve fibers	+++

*In all organs examined: Grading scale: +/− = stained equivocally; + = stained weakly; ++ = stained moderately; +++ = stained intensely.
Data from hemizygous offsprings of a transgenic line Dm1 was used.

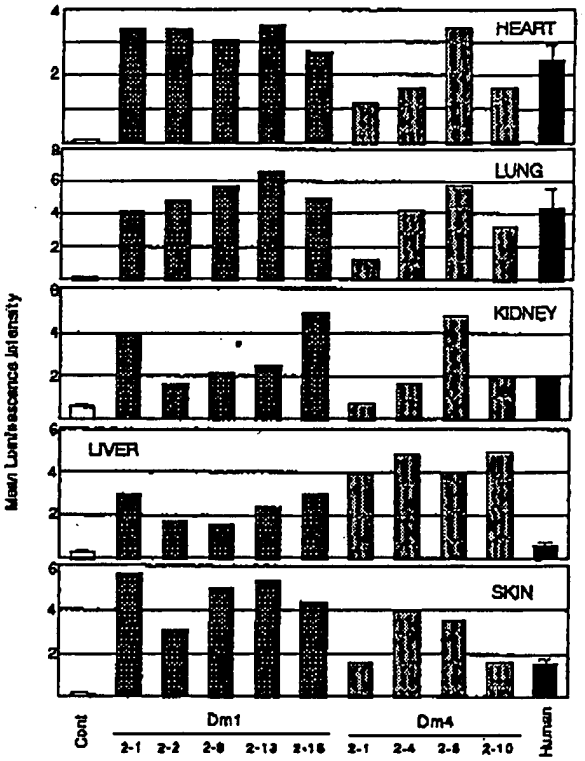

Fig. 3. Comparative analysis of the expression of hDAF by ELISA. An ELISA was performed as described in Materials and Methods. Five organs (heart, lung, kidney, liver, and skin from top to bottom) from nine transgenic and three control non-transgenic descendants from Dm1 and Dm4 lines and a human were used in this study. The results on the transgenic descendants represent the mean value of the measurements in triplicate, and those on the control non-transgenic descendants and human represent mean ± SEM of three independent assessments.

pigs. In the present study, we used a pig endogenous gene promoter to express hDAF in pigs. The pMCP is a complement regulatory protein broadly expressed in pigs like human MCP with some exceptions. For example, the pMCP is expressed on the PRBCs and pancreatic islets where human MCP is not expressed (Bennet et al., 2000). Particularly, the pMCP is intensely expressed on the vascular endothelia (Perez de la Lastra et al., 1999) that are the first target of human natural antibodies and following HAR and AVR. In this regard, we hypothesized that the regulatory elements of the pMCP gene are good candidates of the gene promoters for transgenic pigs useful for xenotransplantation. We used two variants of the pMCP gene promoter region in hDAF gene constructs and obtained transgenic pigs only with a gene construct bearing the 0.9 kb promoter. With 5.4 kb promoter, frequency of live births decreased, suggesting some deleterious effects of the gene on early development in pigs. In our previous studies, transgenic mice were produced in similar frequencies with the two gene constructs (Murakami et al., 2000). The reason for the difference is unclear, but may be dependent upon some species-specific regulatory mechanism. Mosaicism of transgene integration was very frequent in the transgenic pigs. In three of the four transgenic lines, transgenic rates in the second generation wer lower than 30%, indicating a mosaicism in sexual glands. These results coincided with the results of FACS analysis with the PRBCs from the founder pigs. The three founder pigs had only 10%–60% of PRBCs, which were expressing hDAF (data not shown). However, all the PRBCs from piglets in the second generations of all the three lines expressed hDAF.

Expression profiles of hDAF in different tissues from descendants of two transgenic pig lines Dm1 and Dm4 were similar (Table 2), except that the staining intensity was variable in Dm4 descendants. Vascular endothelial walls had particularly high expression in all organs from descendants of both lines. The levels of hDAF on aorta endothelial cells from the transgenic pigs were twice those on human endothelial cells. These results indicate that pMCP promoter is useful to express heterologous proteins in vascular endothelial cells and various other cells in a wide range of organs.

The hDAF expression on PRBCs decreased as the pigs grew older (data not shown). No change of hDAF expression with age was seen in the other tissues or cells such as vascular endothelial cells. Expression levels of ndogenous pMCP on PRBCs in transgenic

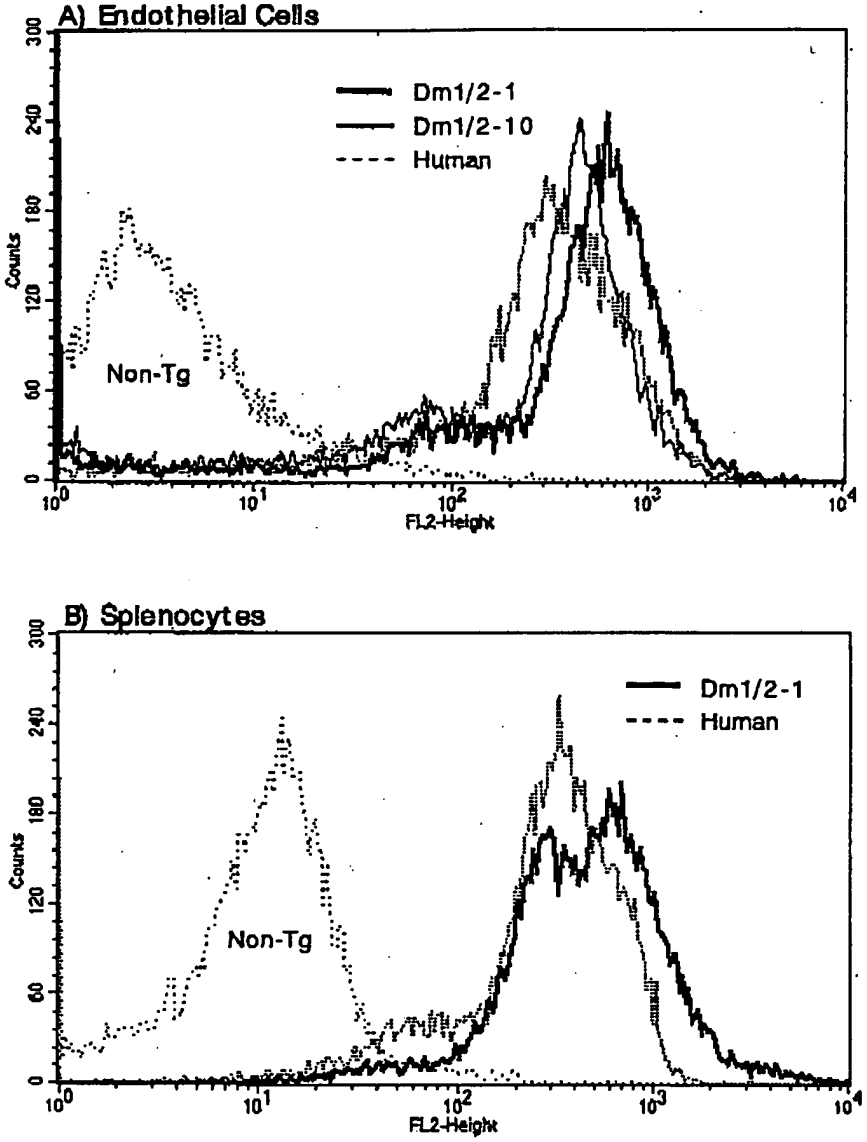

Fig. 4. Flow cytometric analysis of the hDAF expression on the aorta endothelial cells (A) and splenocytes (B) from Dm1 transgenic or non-transgenic descendants. The hDAF expression intensities of the pig cells were compared with HAECs (A) or human peripheral blood lymphocytes (B). HDAF were detected using a biotinylated anti-hDAF mAb (IA10) and streptavidin-conjugated phycoerythrin followed by flowcytometric analysis.

and nontransgenic pigs assessed using anti-pMCP mAb, did not change with age (data not shown). Also, in the transgenic mice previously produced with the same gene constructs, downregulation in the expression of hDAF on PRBCs with age did not occur (unpublished results). Therefore, such downregulation of the transgene expression was dependent upon species and tissues.

Quantities of hDAF protein expressed in different organs varied between the two lines (Fig. 3). In the Dm1 line, the descendants had similar levels of hDAF expression. The expression levels in Dm1 line were greater than those of the corresponding human organs. The descendants in Dm4 line had various expression levels of hDAF. This can be accounted for by two different transgene integration sites as described above. The highest expresser in the Dm4 descendants exhibited as high as Dm1 descendants. Interestingly, liver from low expresser f Dm4 descendants exhibited constantly higher amounts of hDAF than Dm1 descendants with similar staining profiles in immunohistochemical stainings as shown in Table 2.

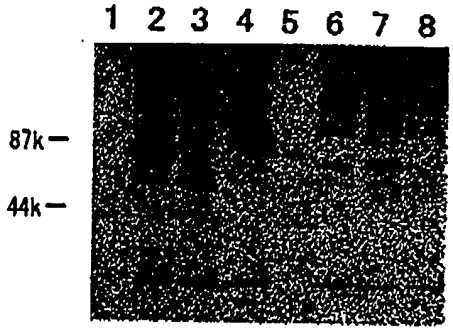

Fig. 5. Western blotting analysis with liver extracts. The liver extracts were concentrated with immunoprecipitation with an anti-hDAF mAb (IIH6) and analyzed by Western blotting using an anti-hDAF mAb VIIIA7 (lanes 2 to 4) or the control irrelevant mAb (lanes 6 to 8). Lanes 1 and 5, molecular markers; Lanes 2 and 6, Dm1 transgenic descendant; Lanes 3 and 7, non-transgenic descendant; Lanes 4 and 8, human PRBCs used as a positive control.

Previous reports suggested that the surface expression of hDAF, but not hMCP, on human cells is upregulated under inflammatory conditions in vitro (Mason et al., 1999). We investigated whether hDAF expression is upregulated in our transgenic pigs with cytokines that exist at sites of inflammation. The cytokine stimulation induced a two-fold increase of the hDAF expression in the human cells as expected, whereas it had no effect on hDAF expression in the transgenic pig cells, suggesting that the pMCP gene may not have a feedback mechanism that responds to the stimulation of cytokines or inflammation. Interestingly, the levels of hDAF on the stimulated human cells were comparable to those of the resting transgenic pig cells (Fig. 6).

Human islet cells express hCD59, but not hMCP or hDAF. On the contrary, porcine islet cells express both pCD59 and pMCP. Previous examination showed that pancreatic islet cells fr m the hDAF transgenic pigs produced with hDAF minigen expressed no or marginal amounts of hDAF (Bennet et al., 2000). We are conducting further examinations to determine the level of hDAF expressed in islet cells from the transgenic pigs produced in this study.

Previous studies suggested that the genetic modifications with genes of complement regulatory factors are effective in preventing HAR, but not AVR in pig-to-primate transplantation (Schmoeckel et al., 1998). For preventing AVR, in addition to control of complement activation, reduction of xenoantigens such as α-Gal in pig organs would be necessary. Recently, we have produced transgenic pig lines expressing a glycosyltransferase; human β-D-mannoside β-1,4-*N*-acetylglucosaminyltransferase III (GnT-III). Xenoantigens in the GnT-III transgenic pigs were remodeled and their cells resisted against both complement- and natural killer cell-mediated pig cell lysis (Miyagawa et al., 2001). We plan to produce transgenic pigs expressing both hDAF and GnT-III by crossing the transgenic pig lines. Such transgenic pigs would contribute to an improvement of the donor pigs for xenotransplantation.

Another problem in xenotransplantation using pigs is a risk of infection with porcine endogenous retroviruses (PERVs) to the human recipients (Patience et al., 1997). As they are present in a high copy number in pig genomes, it may be difficult to eliminate these proviruses by available genetic technologies. However, there is no evidence that patients who have ever been transplanted with pig organs are infected with PERVs (Paradis et al., 1999). Therefore, for the time being, a careful assessment of whether human patients treated with xeno-organs, tissues, or cells are infected with PERVs and pose a risk to other people should be conducted. If PERVs are found not to result in clinical problems in the organ recipients for many years, the

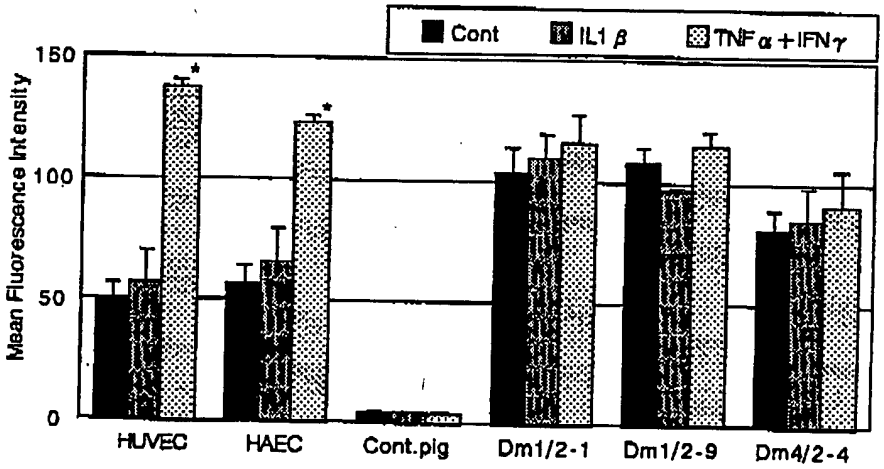

Fig. 6. Analysis of hDAF expression on endothelial cells after stimulation with cytokines. Aorta endothelial cells from three transgenic and one non-transgenic descendants of Dm1 and Dm4 lines and two human cell lines were incubated for 48 hr in the presence or absence of IL-1β or a combination of TNF-α and IFN-γ and analyzed by flow cytometry. The results represent mean ± SEM of three independent experiments, $^{*}P < 0.01$.

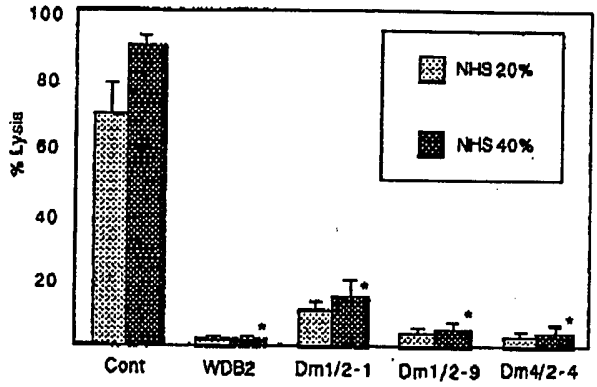

Fig. 7. Human serum-mediated cell lysis assay of endothelial cells from transgenic and non-transgenic pigs. Cells from a control pig and three transgenic descendants of Dm1 or Dm4 lines, and a pig endothelial cell line (WDB2) expressing hDAF two times higher than HUVEC were treated with 20% or 40% of NHS as described in Materials and Methods. Cells from transgenic pigs and WDB2 cells were significantly more resistant than control cells, $^*P < 0.01$.

benefit of a successfully improved xenotransplantation may outweigh the risks of the infection of PERVs.

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